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Immobilization of DNA via Covalent Linkage for Use as Immunosorbent

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DNA was immobilized covalently to Sepharose by several methods using epichlorohydrin, cyanogen bromide, carbodiimide, hydroxysuccinimide, carbonyldiimidazole, trichlorotriazine, and diazonium salt. These immobilizing methods were compared from the standpoint of the preparation of immunosorbent for anti-DNA antibodies. Among these methods, that involving epichlorohydrin was the most suitable because of large coupling capacity, stability of bound DNA, and nonadsorption of anti-DNA by the support itself.

Plasma exchange has been utilized recently as a therapy for autoimmune diseases. However, it has significant disadvantages such as the necessity for the use of fresh plasma and albumin to replace the patient's plasma and the consequent risk of infection. A better therapy for autoimmune diseases would involve the selective removal of pathogens from blood by adsorption (1, 2). In systemic lupus erythematosus, anti-DNA antibodies and their immune complexes play an important role in pathogenesis (3–5). Terman *et al.* described immunosorbents including entrapped DNA (6–8), and El Habib *et al.* described an immunosorbent prepared by immobilization of DNA onto an acyl-azide derivative of collagen (9). The immunosorbent-linked DNA is expected to show selective adsorption of anti-DNA. Such immunosorbents should have large binding capacity for antibodies, should not exhibit nonspecific adsorption, and should not release ligands or other chemicals. In view of the above demands, DNA should be attached tightly to the support via the formation of a stable covalent linkage.

A variety of alternative techniques have been developed to immobilize biological materials, and for DNA, many methods were attempted (10–13). But a comparison of immobilizing procedures for the preparation of immunosorbents seldom has been investigated. We have studied the immobilization of DNA by several covalent methods from the standpoint of degree of immobilization, stability of ligands, and specificity of adsorption. This investigation was performed using Sepharose as a support because of its high porosity, chemical stability, and inertness.

MATERIALS AND METHODS

Materials. Sepharose 4B and CL-4B were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. DNA from salmon sperm, epichlorohydrin, and 2,4,6-trichloro-1,3,5-triazine (TCTA)[1] were from Wako Chemicals Industries, Ltd., Osaka, Japan.

[1] Abbreviations used: TCTA, 2,4,6-trichloro-1,3,5-triazine; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; DCC, *N,N'*-dicyclohexylcarbodiimide; CDI, 1,1'-carbonyldiimidazole; DMF, *N,N*-dimethylformamide; PBS, 0.02 *M* phosphate-buffered saline (pH 7.35).

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Cyanogen bromide and *N*-hydroxysuccinimide were obtained from Nakarai Chemicals, Ltd., Kyoto, Japan. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC) was from Aldrich Chemical Company, Milwaukee, Wisconsin. *N,N'*-Dicyclohexylcarbodiimide (DCC) and 1,1'-carbonyldiimidazole (CDI) were from Tokyo Kasei Kogyo Company, Ltd., Tokyo, Japan. Quantafluor Fluorescent Autoantibody Test using *Crithidia luciliae* substrate and Quantafluor anti-native DNA positive control (human serum with anti-native DNA activity) were from Kallestad Laboratories, Inc., Austin, Texas. All solvents were of analytical grade.

The Sepharose beads were washed with water and sucked to a moist cake prior to use. Sepharose 4B was used when activation was performed in water, and Sepharose CL-4B was used in organic solvents. The dry weight of beads was measured after lyophilization. Absorbance was measured with a Hitachi 220A spectrophotometer.

Reaction with oxirane groups (Method A). Epichlorohydrin-activated Sepharose 4B was prepared according to the procedure of Matsumoto *et al.* (14), and oxirane rings in the gel were determined by the method of Sundberg and Porath (15). The activated beads (5.0 g of moist cake, corresponding to about 230 mg dry wt) were mixed with 10 ml of DNA solution whose pH was adjusted with a buffer. The suspension was shaken under the conditions shown in Table I. After the reaction, the mixture was transferred on a glass filter and washed twice with 20 ml of 0.1 *M* acetate buffer (pH 4.0) containing 0.5 *M* NaCl and 20 ml of 0.2 *M* carbonate buffer (pH 9.0), and washed with 40 ml of 0.1 *M* borate buffer (pH 7.3), 40 ml of 1 *M* NaCl, and finally with 1 liter of water.

Immobilization via CNBr activation in carbonate buffer (Method B-1). Activation of Sepharose 4B (5.0 g) and determination of cyanate esters were performed as described by Kohn and Wilchek (16). The activated beads were washed with cold coupling buffer and cold water and shaken in DNA solution. The reaction mixture was filtered and washed with water. The resultant beads were shaken in 10 ml of 0.2 *M* glycine (pH 8.7) at room temperature for 2 h. The beads were filtered off and washed as described in Method A.

Immobilization via CNBr activation in phosphate buffer (Method B-2). Sepharose 4B (5.0 g) was suspended in 10 ml of 3.75 *M* potassium phosphate buffer (pH 12). Cyanogen bromide (1 g) in *N,N*-dimethylformamide (DMF, 1 ml) was poured into the mixture which was vigorously shaken below 10°C for 3 min. The immobilization of DNA was performed as described in Method B-1.

Succinoylation of Sepharose. Sepharose CL-4B (93.2 g, 3.66 g dry wt) was successively washed on a glass filter with water:dioxane (1:1), dioxane, and dry dioxane, and then suspended in dry dioxane (200 ml). Dry pyridine (20 ml) and succinic anhydride (11.8 g) were added and the mixture was shaken at room temperature for 5 h. The beads were filtered off and washed with dioxane, acetone, water, and 0.1 *M* acetic acid, and finally washed with water until the eluate was neutral. Carboxyl groups in the gel were determined by titration.

Immobilization using CMC (Method C). Succinoyl-Sepharose CL-4B (5.0 g, 0.24 dry wt, 0.98 mmol carboxyl groups/g dry wt) was mixed with DNA solution (10 ml) and CMC (0.98 g). After the reaction, the beads were treated as described in Method A.

Conversion of succinoyl-Sepharose to an active ester and condensation with DNA (Method D). Succinoyl-Sepharose CL-4B (22 g, 0.93 g dry wt, 1.13 mmol carboxyl groups/g dry wt) was gradually transferred to dry dioxane as previously described. The beads were suspended in dry dioxane (80 ml), and *N*-hydroxysuccinimide (0.98 g) and

DCC (1.76 g) were added. The mixture was shaken at room temperature for 2 h, filtered, and washed with dioxane, acetone, and water. The activated beads (5.0 g, 0.72 g dry wt) were mixed with DNA solution (10 ml) and the mixture was shaken at 4°C for 15 h. The reaction mixture was filtered and washed with water. The resultant beads were treated with 10 ml of 1 *M* glycine (pH 9.0) at room temperature for 15 h while shaking. The beads were filtered off and washed as described in Method A.

Reaction with CDI (Method E). Sepharose CL-4B (30 g) was transferred to dry dioxane as described before and suspended in dry dioxane (100 ml). CDI (6.8 g) was added and the suspension was shaken at room temperature for 5 h. The mixture was filtered and washed with dioxane, dioxane:water (1:1), and water. Imidazoylcarbonate esters in the beads were calculated from the nitrogen content. The activated beads (5.0 g, 0.55 g dry wt) were shaken in DNA solution. The following operations were the same as described in Method D.

Immobilization via TCTA activation (Method F). The activation of Sepharose CL-4B was performed by the method of Smith and Lenhoff (17). The activated beads (5.0 g, 243 mg dry wt) were shaken in DNA solution. The treatment after the reaction was the same as in Method D.

Immobilization via diazonium salt (Method G). Sepharose CL-4B (136 g, 5.58 g dry wt) was washed with DMF:water (1:1), DMF, and finally with dry DMF. The beads were mixed with dry DMF (150 ml), pyridine (30 ml), and *p*-nitrobenzoyl chloride (25 g). The mixture was shaken at room temperature for 5 h, filtered, and washed with acetone and water. The resultant white cake was suspended in 0.2 *M* NaCl (300 ml) and the mixture was shaken at 50°C for 1 h after addition of sodium dithionite (70 g). The pale yellow product was filtered off and washed with 0.2 *M* NaCl and water. The content of amino groups was calculated to be 3.17 mmol/g dry wt from the nitrogen content. The *p*-aminobenzoyl-Sepharose (40 g, 5.2 g dry wt) was poured into 0.1 *M* HCl–0.2 *M* NaCl solution (800 ml). The mixture was cooled at 4°C, and 1 *M* $NaNO_2$ (80 ml) was added. After 10 min of shaking, the mixture was filtered and the reddish brown cake was washed with cold 0.2 *M* NaCl and cold water. The activated beads (5.0 g, 0.93 g dry wt) were shaken in DNA solution. After the reaction, the beads were treated as described in Method D.

Determination of bound DNA. Bound DNA was calculated by the phosphorus content which was determined in the following manner. A sample (0.2 g of moist beads) was lyophilized and ashed by heating with 0.2 ml of concentrated H_2SO_4 and 0.2 ml of 70% $HClO_4$. The resultant colorless solution was exactly diluted with water and the phosphate was assayed by the colorimetric method of Chen *et al.* (18) using KH_2PO_4 as standard. The phosphorus in dry DNA was 9.14 wt%.

Bound DNA was also determined by the color development of deoxyribose. A sample (0.1–0.2 g) was mixed with 50% $HClO_4$ (2 ml) at room temperature. The suspension gave a clear solution within 5 h. Then this solution was diluted to 25 ml with water and the DNA was determined by the method of Burton (19). The DNA concentration was calculated by measuring the 600–700 nm optical density difference, which was directly proportional to the phosphorus content.

Adsorption of anti-native DNA. A sample (50 mg of beads), which was washed with 0.02 *M* phosphate-buffered saline (PBS, pH 7.35), was mixed with PBS (175 μl) and an anti-native DNA positive control (75 μl). The mixture was shaken at room temperature for 1 h and centrifuged at 1000*g* for 5 min. The concentration of anti-native DNA in the supernatant was determined by means of the Quantafluor Fluorescent Autoantibody Test, which is an indirect fluorescent antibody procedure. The fluores-

cence of the kinetoplast of the *C. luciliae* substrate was observed at 500 magnification with an Olympus BHS-RF-A microscope with fluorescence optics. The intensity of uorescence was divided into five grades: +++, ++, +, +−, and −.

RESULTS AND DISCUSSION

The results of immobilization experiments are summarized in Table I. The amount of bound DNA was determined mainly by the phosphorus content of the conjugates. However, when activation or treatment of activated beads was performed in a phosphate buffer (cyanogen bromide method), the resultant beads contained appreciable amounts of phosphorus. Therefore, when a phosphate buffer was used during activation

TABLE I

Results of DNA Immobilization[a]

Method	*Buffer*	*pH of DNA solution*	*Temperature (°C)*	*Time (h)*	*Bound DNA (mg/g dry support)*
A[b]	0.2 M Carbonate	10.6	25	24	89.7
A	0.2 M Carbonate	9.9	25	24	52.4
A	0.2 M Carbonate	8.9	25	24	25.0
A	0.2 M Carbonate	7.0	25	24	19.7
B-1[c]	0.2 M Carbonate	10.0	20	20	5.8
B-1	0.2 M Carbonate	8.1	20	20	7.8
B-1	0.4 M Borate	6.6	20	20	7.2
B-1	0.4 M Borate	4.5	20	20	8.6
B-1	0.2 M Citrate	4.1	20	20	12.1
B-2[c]	0.2 M Carbonate	10.0	20	18	15.1
B-2	0.2 M Carbonate	9.2	20	18	31.9
B-2	0.2 M Borate	7.8	20	18	49.2
B-2	0.2 M Phosphate	6.0	20	18	46.7
B-2	0.2 M Phosphate	5.0	20	18	62.7
C	0.4 M Borate	7.2	0–10	24	4.7
C	0.4 M Borate	6.6	0–10	24	15.3
C	0.4 M Borate	5.5	0–10	24	48.6
C	0.4 M Borate	4.6	0–10	24	39.1
D	0.2 M Phosphate	7.8	4	15	2.9
D	0.2 M Phosphate	6.7	4	15	2.5
D	0.2 M Phosphate	5.1	4	15	1.1
E[d]	0.2 M Phosphate	6.8	25	22	1.5
E	0.2 M Acetate	5.3	25	22	2.5
E	0.2 M Acetate	4.5	25	22	3.4
F	0.2 M Carbonate	10.2	4	2	5.1
F	0.2 M Carbonate	8.4	4	2	3.4
F	0.2 M Borate	6.8	4	2	2.8
G	0.4 M Borate	10.2	4	24	6.6
G	0.4 M Borate	8.8	4	24	4.6
G	0.4 M Borate	7.6	4	24	7.9

[a] Each experiment was performed with 5.0 g of moist beads and 10 ml of DNA solution (18.6–19.0 mg/ml of DNA). Symbols of methods are the same as used under Materials and Methods.

[b] Oxirane groups in activated beads were 0.72 mmol/g dry wt.

[c] Cyanate esters were 0.44–0.57 (B-1) and 1.0–1.1 mmol/g dry wt (B-2) when activation was performed under the same conditions.

[d] Imidazolylcarbonyl groups in activated beads were 0.37 mmol/g dry wt.

or immobilization, bound DNA was determined by another method (see Materials and Methods).

The immobilization was performed at different pH values. The pH of immobilization strongly affected the amount bound in almost all methods. In Methods A and F, the amount of bound DNA increased with increasing pH. This result is expected because the reaction site of DNA is probably the amino groups of bases in the region where the double-stranded chain is loosened. In contrast with this, the amount of bound DNA increased with decreasing pH in Methods B-1, B-2, and E. Reaction with CNBr-activated resin has usually been carried out under neutral or basic conditions. It is interesting and has not been reported previously that the optimum pH of coupling is in the acidic area. For example, the optimum pH for methionine binding is around 8 (20). CNBr-activated beads seemed to react with the phosphate ion as described above. These results suggest that terminal phosphate groups of DNA are concerned in immobilization to CNBr-activated beads. The difference in the results from Methods B-1 and B-2 seems to depend mainly on the amount of reactive groups in the gel. In Method C, the maximum bound DNA was obtained at pH 5.5. This is explained as follows. Possible intermediates are formed by the reaction of the CMC with the carboxyl groups of the resin and with the phosphate groups of DNA. Both intermediates are expected to be hydrolyzed rapidly under acidic or basic conditions.

The order of the yield of immobilization may be concluded to be Method A > B-2 > C > B-1 > the others.

The stability of bound DNA on storage was tested. After storage at 4°C, the remaining DNA was measured and Fig. 1 shows the results. Method A was the best with respect to the stability of bound DNA among all methods examined. In Method A, the linkage between Sepharose and DNA consists of ether linkages and alkyl carbon-amino nitrogen bonds. Both kinds of bonds are chemically stable.

Finally, the adsorption of anti-native DNA antibodies was examined by the use of the following supports: Sepharose 4B, modified Sepharose, Sepharose–DNA conjugates, and Sepharose which was activated and treated under the same conditions of im-

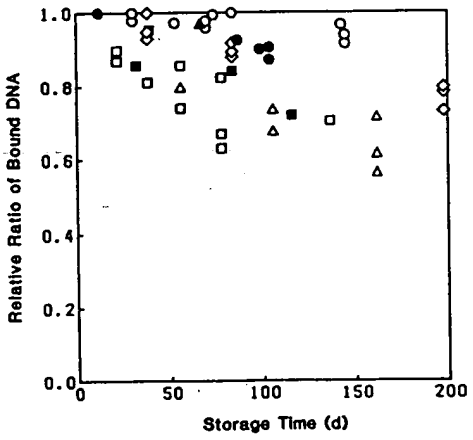

FIG. 1. DNA release from the conjugates during storage at 4°C. After the storage in water containing 0.1% NaN_3, the beads were washed with 1M NaCl and water, and bound DNA was measured. Relative ratio of bound DNA per dry support was plotted against the storage time. Immobilization method: ○, A; □, B-1; ◇, B-2; △, C; ■, E; , F; ▲, G.

TABLE II

Adsorption of Anti-native DNA[a]

Treatment of Sepharose[b]	*DNA in sample (mg)*	*Anti-DNA in supernatant*
None[c]	None	+++
Activated (A)[d]	None	+++
Activated (A)[e]	None	+++
Immobilized (A)	0.05	++
Immobilized (A)	0.17	+−
Activated (B-1)[e]	None	++
Immobilized (B-1)	0.02	++
Activated (B-2)[e]	None	+
Immobilized (B-2)	0.14	−
Succinoylated[f]	None	++
Immobilized (C)	0.14	+−
Activated (F)[e]	None	−
Aminobenzoylated[g]	None	−
Activated (G)[e]	None	−

[a] The interpretations of anti-native DNA at 2-, 4-, 8-, 16-, 32-, 64-, and 128-fold dilution with PBS were +++, +++, ++, +, +, −, and −, respectively.
[b] The symbols in parentheses are those of activating methods.
[c] Sepharose 4B.
[d] Sample beads contained 2.3 μmol of oxirane groups.
[e] The beads were treated under the same conditions of immobilization without DNA.
[f] Sample beads contained 2.0 μmol of carboxyl groups.
[g] Sample beads contained 23 μmol of amino groups.

mobilization without DNA (Table II). When the supports contained no DNA, the adsorption of anti-DNA was not observed in Sepharose and oxirane-activated Sepharose, but was slightly observed in CNBr-activated Sepharose and succinoyl-Sepharose, and was strongly observed in aminobenzoyl-Sepharose and TCTA- and diazonium-activated Sepharose. The support, which itself adsorbs antibodies, is not suitable as a selective immunosorbent. At this point, Method A is also the best, because the spacer is hydrophilic and not charged. As expected, DNA–Sepharose conjugates showed adsorption of anti-DNA.

As described above, the oxirane method is the best immobilizing procedure of the six methods examined because the amount of bound DNA is large, the bound DNA is stable on storage, and nonspecific adsorption of anti-DNA by the support is negligible. We plan to describe our further investigations of DNA immobilization by the oxirane method.

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